Makgatho et al., Afr J Tradit Complement Altern Med., (2017) 14 (5): 181-187
https://doi.org/10.21010/ajtcam.v14i5.21

EVALUATION OF THE ANTI-MYCOBACTERIAL, ANTI-OXIDATIVE AND ANTI-PROLIFERATIVE ACTIVITIES OF *RUBIA CORDIFOLIA* ETHANOLIC LEAF EXTRACT SUB-FRACTIONS IN HUMAN LYMPHOCYTES AND NEUTROPHILS

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Article History

Received: 26, April. 2017

Revised Received: 6, June. 2017

Accepted: 6, June. 2017

Published Online: 01, Oct. 2017

Abstract

Background. *Rubia cordifolia* has been used to treat various diseases and exhibits antimicrobial, anti-inflammatory, analgesic and antidiarrheal activities. The plant has not been investigated for its anti-mycobacterial activity against virulent tuberculosis strains as well as cytotoxic and anti-oxidant activities in mononuclear cells and neutrophils.
Methods. Ethanolic and dichloromethane leaf extract fractions of *Rubia cordifolia* (0.2- 125µg/ml) were screened for anti-mycobacterial activity using a fluorescent microplate assay. The anti-oxidant activity of the fractions was tested using the radical scavenging assay, while their cytotoxicity to lymphocytes was measured using the WST-8 assay. Reactive oxygen species production by neutrophils exposed to fractions was tested using Cell Meter™ Fluorimetric ROS Assay kit.
Results. The ethanolic leaf fractions exhibited overall superior anti-mycobacterial activity than the dichloromethane group and were further screened for anti-oxidant and cytotoxic activities. Ethanolic sub-fractions K2F-3.2, K2F-3.3 and K2F-3.4 showed significant antioxidant activities at concentration of 50 µg/ml to 200 µg/ml and higher in both the cell free-based radical scavenging activity and total reactive oxygen species production assays. For lymphocytes, the test agents showed anti-proliferative activity at 25 µg/ml to 200 µg/ml for sub-fraction K2F-3.3B and 50 µg/ml to 200 µg/ml for agents K2F-3.2 and K2F-3.4.
Conclusion. The current study is the first to record the *in vitro* anti-mycobacterial, anti-oxidant and cytotoxic activities of *Rubia cordifolia* plant leaf extract sub-fractions using the given experimental setups and further research activities to identify the bioactive components are to be pursued.

Key words: *Rubia cordifolia*, DPPH, Mycobacteria, cell proliferation, antioxidant activity, ROS.

Introduction

Tuberculosis (TB), compared to HIV/AIDS, is the second highest communicable disease that leads to high mortality rates worldwide due to *Mycobacterium tuberculosis* (WHO, 2014; WHO, 2015). The disease is expected to surge in the future due to various forms of multidrug drug resistance (MDR): multidrug resistant (MDR), extremely drug resistant and (XDR) and totally drug resistant (TDR) tuberculosis and co-infection with human immunodeficiency virus (HIV) (Mistry et al., 2012; Abubakar et al., 2013 and WHO, 2015). The South African TB/HIV/AIDS co-infections are extremely high with 73% of TB patients co-infected with HIV (Soul City Research Unit, 2015). This situation calls for an emergence prioritisation of development of new and novel agents for TB treatment and management.

There is a resurgence of research activities to explore the use of medicinal plant bio-products as potential anti-mycobacterial agents in South Africa (Lall and Meyer, 1999; Bapela et al., 2006; Eldeen and Van Staden, 2008; Green et al., 2010; Labischagné et al., 2012; Aro et al., 2016 and Komape et al., 2017). Plant extracts with high anti-oxidant capacity have been presented as exhibiting bactericidal activity against *Mycobacterium tuberculosis* (Newton et al., 2000; Okunade et al., 2004; Pauli et al., 2005 and Gouveia-Figueira et al., 2014; Magadula et al., 2012). Tuberculosis as an infection present an inflammatory response in the lungs whereby cellular mediators are released causing oxidative stress (Sommer, 2005). The most vital of the mediators are reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2). Excess production of these oxidants leads to harmful inflammatory reactions in the lungs (caseous necrosis) and antioxidant material that scavenges and eliminate ROS may be important in preventing or minimizing oxidative-

related damage in clinical conditions like tuberculosis (Constantino et al., 1992; Reljic et al., 2010; Bernardes et al., 2014 and Dzoyem and Ellof, 2015).

Other studies have focused on the repurposing of compounds that have anti-inflammatory activity as they present an alternative to combating increased antimicrobial drug resistance (Kulkarni and Deshpande, 2016; Maitra et al., 2016 and Prasad et al., 2016). Plant products or chemical entities that exhibits antimicrobial and anti-inflammatory activities should be considered for prospects in combatting *Mycobacterial tuberculosis* infection (Trevisan et al., 2016). *Rubia cordifolia* Linn. (Rubiaceae) is also known by other common names like Manjistha and Majith depending on the geographical location. It is distributed in tropical Africa, India, Japan and Indonesia (Tailor et al., 2010; Deshkar et al., 2008). The various parts of the plant have been shown to exhibit a myriad of ethno-medicinal applications like: anti-diarrheal, anti-inflammatory, antimicrobial, antipyretic and analgesic activities (Kasture et.al 2000; Joharapurkar et.al., 2003; Devi Priya and Siril., 2014; Verma et.al., 2016; Gong et.al., 2017). Medicinal plants and their bioactive compounds can serve as an alternative choice for resistant Tuberculosis treatment, provided the agents are less toxic and anti-inflammatory because Tuberculosis is a highly inflammatory phenomenon that damages lungs (Semenya and Maroyi, 2013; Nyambuya et al., 2017; Komape et al., 2017).

To this end, the anti-mycobacterial activity of crude ethanolic and dichloromethane (DCM) leaf extracts of *Rubia cordifolia* were investigated for anti-mycobacterial activity *in vitro*. The crude extracts that exhibited the most potent activity were further fractionated to screen for possible: anti-mycobacterial, antioxidant and anti-proliferative activities.

Materials and Methods

Unless otherwise indicated, all chemicals and reagents were obtained from Sigma-Aldrich (Johannesburg, South Africa).

Plant collection and preparation of leaf extracts and sub-fractions

Rubia cordifolia plants were collected from traditional healers in Tzaneen (Mopani District, Limpopo Province, South Africa) and a voucher specimen of the plant was deposited in the University of Limpopo Herbarium under the accession number 11208. Ethanol (100 ml) was added to 5 g of dried powder from plant leafs in a 500 ml volumetric flask. The resultant mixture was stirred at room temperature for 24 h. The mixture was filtered to remove undissolved material and then washed with 2 x 10 ml Ethanol. The solvent was removed in a rotary evaporator to give 2.233 g of crude extract. The crude extract was then subjected to preparative thin layer chromatography (prep-TLC), eluting with methanol-DCM (1:19) to give four fractions.

Green fluorescent protein microplate assay (GFPMA) for anti-mycobacterial screening

This broth micro dilution technique (Collins et. al., 1998) allows an array of drug concentrations to be screened on a single 96-well microtitre plate in order to determine minimum inhibitory concentration (MIC). Briefly, culture (10 ml) of a mutant *Mycobacterium tuberculosis* (H37RVMA) strain (Ioerger et.al., 2010) constitutively expressing recombinant green fluorescent protein (GFP) is grown to an OD_{600} of 0.6-0.7 and diluted 1:100 in GAST/Fe medium. To the wells of the test plate was added 100 µl of culture suspension and two-fold dilutions of the test agents (0.2-125 µg/ml, final concentration) in triplicates to a final volume of 200 µl. Control wells contained untreated bacterial culture suspensions (positive control) and medium only (background control). The plates were incubated at 37^{o}C and fluorescence measured at day 7 post incubation using a Flurostar Optima microplate reader (BMG Labtech, Thermofisher Scientific, USA) with excitation at 484 nm and emission at 508 nm. Percent inhibition was calculated as 1- (test well FU/FU of background wells X 100). MIC was expressed as the lowest drug concentration inhibiting 90% and 99% of bacterial growth.

Determination of antioxidant activity of ethanolic sub-fractions by DPPH method

The radical scavenging activity of the extract was determined by the method described by Krings and Berger (Krings and Berger, 2001). To 1 ml of 0.1 mM solution of DPPH in ethanol, was added 3 ml of test agents (6-200µg/ml) in distilled water and kept for 30 minutes. The colour change of the reaction mixture was then read at 517 nm in a plate reader DXT 800 multimode plate reader (Beckman Coulter, Johannesburg, South Africa).

Preparation of PBMCs from human peripheral blood

PBMCs were obtained by collecting venous blood (8ml) into specialised BD Vacutainer® CPT™ tubes according to the manufacturer's instruction (Beckton Dickinson, Johannesburg, South Africa). Ethical approval to draw blood from donors was obtained from the University of Limpopo Research and Ethics Committee (TREC/117/2016:UG).

Isolation of neutrophils from human peripheral blood

Purified neutrophils were prepared from heparinized venous blood of healthy adult human volunteers and were separated from mononuclear leukocytes by centrifugation on Histopague-1077 at 300 g for 25 min at 4^0C. After removal of the plasma and mononuclear cell layers, 5ml of Verasylase was added to the residual red blood cell pellet that contained neutrophils and incubated for 15 min to lyse the erythrocytes. After incubation, the suspension was resuspended in abundant HBSS buffer and centrifuged at 160 g for 10 min. The supernatant was aspirated and the pellet washed twice in HBSS at 110 g for 10 min. Neutrophils of high purity (>90%) were prepared at 2 x 10^7cells/ml and held on ice before assaying for total ROS production.

Detection of total ROS production by ethanolic sub-fractions on purified neutrophils

Cells were incubated for 5 min with or without test material (6-200 µg/ml) and ROS production detected using a Cell Meter™ Fluorimetric ROS Assay Kit according to the manufacturer's instruction (Cayman Chemical, Johannesburg, South Africa).

Determination of growth inhibitory effects of ethanolic sub-fractions on human lymphocytes

After incubating the PBMCs (2 x 10^6 cells/ml) for 72 hours with or without plant isolates (6-200 µg/ml), proliferative activity was determined using the WST-8 assay following the manufacturer's instructions (Cayman Chemicals, Johannesburg, South Africa).

Data analysis

Numerical data of experiments (repeats of 3-4 experiments in triplicates) was analysed using Microsoft Excel 2009 Version for Windows. Results are expressed as mean ± SD (Standard deviation). A $p \leq 0.05$ considered to be significant.

Results

Anti-mycobacterial activity of *Rubia cordifolia* leaves were tested on DCM and ethanolic extracts against H_{37}Rv strain of *Mycobacterium tuberculosis* using the green fluorescence protein microplate assay (GFPMA). The ethanolic crude extract (K2F-3) was less active than the DCM (K2F-1) crude extract, with MIC_{90} of 57.3 and 44.5 µg/ml respectively as shown in Table 1 and Table 2 respectively. Fractionation of the crude extracts by preparative chromatography, eluting with 5% methanol-DCM afforded four sub-fractions from each crude. The sub-fractions from the ethanol crude demonstrated better activity compared to those obtained from the DCM crude (Table 1 and 2) and were further screened for anti-oxidant activity.

Table 1: Minimum inhibitory concentrations (MIC_{90} and MIC_{99}) of ethanolic leaf extracts of *Rubia cordifolia* against the H37Rv strain of *Mycobacterium tuberculosis*.

Sample name	MIC_{90} (µg/ml)	MIC_{99} (µg/ml)
K2F-3 (Ethanol crude)	57.3	63.7
K2F-3.1	35.5	41.2
K2F-3.2	26.2	28.7
K2F-3.3	38.8	45.8
K2F-3.4	39.4	41

Table 2: Minimum inhibitory concentrations (MIC_{90} and MIC_{99}) of DCM leaf extracts of *Rubia cordifolia* against the H37Rv strain of *Mycobacterium tuberculosis*.

Sample name	MIC_{90} (µg/ml)	MIC_{99} (µg/ml)
K2F-1 (DCM crude)	44.5	57
K2F-1.1	52.8	57.8
K2F-1.2	71.6	82.7
K2F-1.3	40.2	46.9
K2F-1.4	27.5	30.2

The DPPH radical scavenging activity measures the antioxidant potential of the ethanolic sub-fractions of *Rubia cordifolia* as shown in Figure 1. The results show that sub-fractions K2F-3.2 B, KF-3.3B and K2F-3.4B exhibits significant DPPH activity (more than 60%) from 50 µg/ml to 200 µg/ml ($p < 0.05$) with sub-fraction K2F-3.1B showing no activity at the concentrations tested ($p > 0.05$). A $p < 0.05$ value also indicates a comparable and significant DPPH activity by the ethanolic crude extract as is Vitamin B_{12} which acted as the positive control in this instance. The three sub-fractions exhibiting significant DPPH activity were then further screened for total ROS production and cytotoxic activity in isolated neutrophils and lymphocytes.

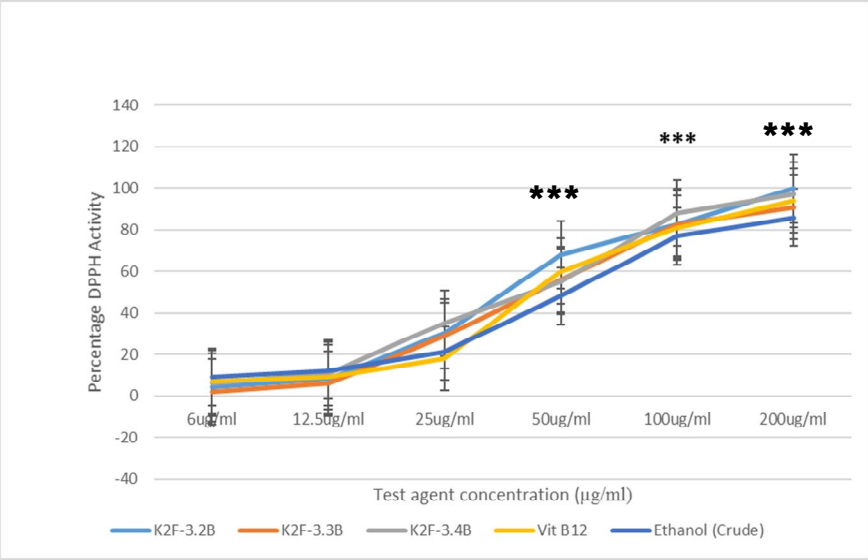

Figure 1: The free radical scavenging activity of ethanolic leaf extract sub-fractions of *Rubia cordifolia* as determined by the DPPH assay. Results are expressed as mean percentage DPPH activity by the test agents (6-200 µg/ml) plus SEM of four different experiments conducted in triplicates. *****The result is statistically significant.

Results for the production of total reactive oxygen species (ROS) by human neutrophils using the Cell Meter™ Fluorimetric ROS Assay Kit are shown in Figure 2. There is an indication of a dose-dependent increase in total ROS production by the three sub-fractions, with sub-fraction K2F-3.3B exhibiting significant activity from 25 µg/ml to 200 µg/ml ($p < 0.05$) while sub-fractions are active from 50 µg/ml to 200 µg/ml ($p < 0.05$).

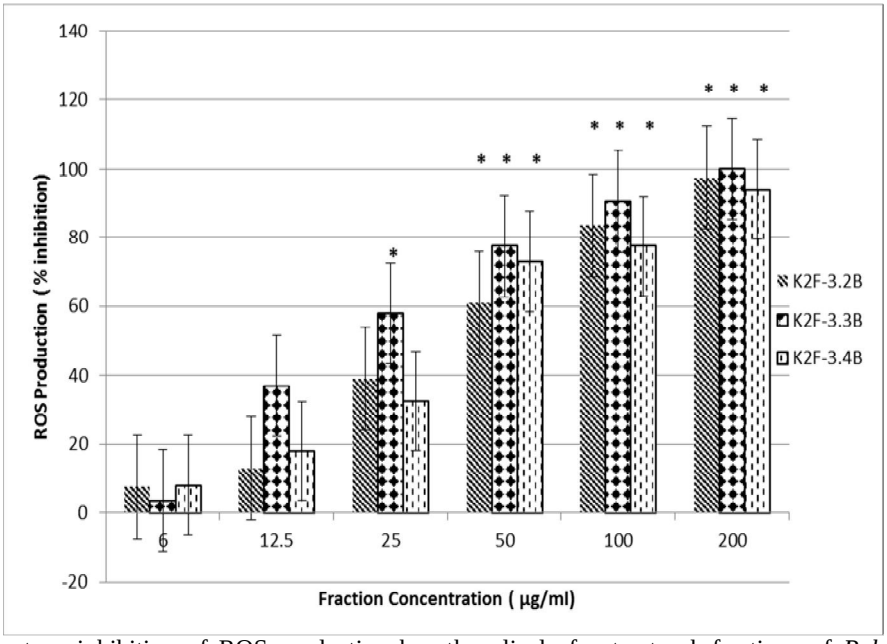

Figure 2: Percentage inhibition of ROS production by ethanolic leaf extract sub-fractions of *Rubia cordifolia* in neutrophils *in vitro*. Results are expressed as the mean percentage of inhibition of ROS production by the test agents (6-200 µg/ml) plus SEM of four different experiments conducted in triplicates. *****The result is statistically significant.

The cytotoxic activity of the three ethanolic sub-fractions of *R. cordifolia* against peripheral blood mononuclear cells (lymphocytes) using the WST-8 assay are indicated in Figure 3. Ethanolic fraction K2F-3.3B did show a significant inhibition of lymphocyte proliferation from 25 µg/ml and higher ($p < 0.05$) while the other two agents, K2F-3.2B and K2F-3.4 exhibited observable cytotoxic activity from 50 µg/ml and higher ($p < 0.05$) as shown in the figure.

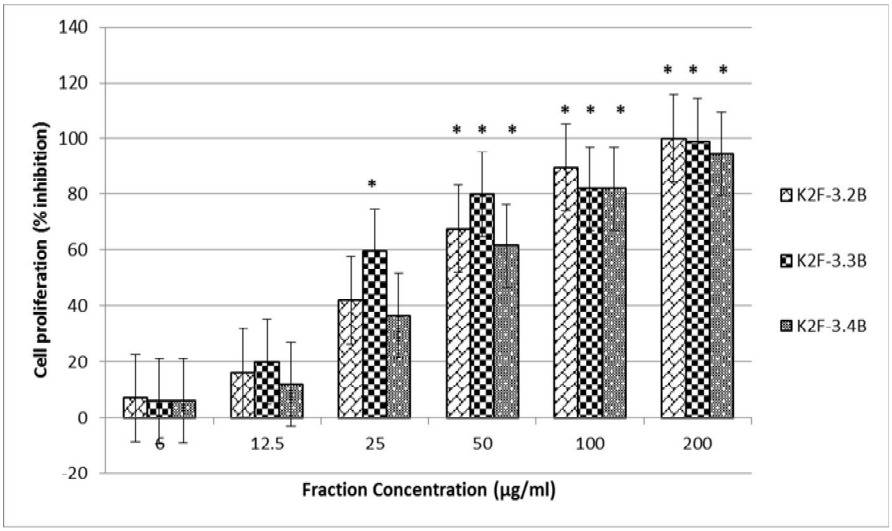

Figure 3: Percentage cell proliferation inhibition by ethanolic leaf extract sub-fractions of *Rubia cordifolia* on lymphocytes *in vitro*. Results are expressed as the mean percentage inhibition of cell proliferation by test agents (6-200 µg/ml) plus SEM of four different experiments conducted in triplicates. *****The result is statistically significant.

Discussion and Conclusion

The current study present results of ethanolic leaf extract sub-fractions of *Rubia cordifolia* showing superior antimycobacterial activity to that of the DCM sub-fractions at IC_{90} values of less than 50 µg/ml compared to that of the DCM test material (highest IC_{90} value of 72 µg/ml). The concentration range attained for *in vitro* anti-mycobacterial activity in this study is comparable or lower to other studies recently reported for different plant and mycobacterial species as well as experimental procedures employed (Bernadres et.al., 2016; Nguta et.al., 2016; Trevisan et.al, 2016; Komape etal., 2017; Nyambuya et.al., 2017).

The other part of the investigations focused on screening the antioxidant and anti-proliferative activities of the ethanolic leaf extracts sub-fractions. Pro-inflammatory mediators play a significant part in protective immunity in tuberculosis and their maintenance and strict control is important to avoid immunopathic cases due to excessive and inappropriate production (Lyadova and Panteleev, 2015; Amaral et.al., 2016; Piñeros et.al., 2017). Only three of the sub-fractions: KF2-3.2B, K2F-3.3B and K2F-3.4 exhibited dose-dependent antioxidant and anti-proliferative activities that attained prominence from 50 µg/ml to 200 µg/ml. There is apparently paucity of literature reports on the screening of leafs extracts of *Rubia cordifolia* in laboratory experimental models used in the current study.

Various studies conducted to investigate the anti-inflammatory and antioxidant potential of *Rubia cordifolia* used roots and stems as screening material (Patil et.al., 2006; Basu and Hazra, 2006; Karodi et.al., 2009; Deoda et al., 2011). The laboratory experimental systems used for anti-inflammatory activity are *ex vixo* and mostly animal models (Lopez-Exposito et.al., 2011; Gong et.al, 2017). The present study affirms that leaf extract sub-fractions of *Rubia cordifolia* does exhibit cytotoxic and antioxidant activities at acceptable concentrations even when different laboratory experimental models are used. In this case we used isolated human peripheral blood mononuclear cells (lymphocytes) and neutrophils. The anti-proliferative and antioxidant properties of leafs of *Rubia cordifolia* are consistent with them containing various chemical derivatives of anthraquinones (Devi Priya and Siril, 2014; Sreenu et.al., 2014; Maharjan and Nampoothiri, 2015; Panigrahi et.al., 2016; Verma et.al., 2016).

The anti-mycobacterial, cytotoxic and antioxidant activities of three ethanolic leaf extract sub-fractions of *Rubia cordifolia* are still preliminary and further laboratory work is warranted to: isolate their bioactive elements, elucidate their apoptotic pathways and screen various drug combination schema in *in vitro* and animal models of experimental Tuberculosis.

Acknowledgements

The project was funded by the University of Limpopo and National Research Foundation (NRF) grant number: **TTK1205280912.**

Ethical consideration: The research proposal and laboratory work were approved by the University of Limpopo Research and Ethics Committee with approval certificate number: **TREC/117/2016:UG.**

Conflict of Interest: Authors declare that there is no conflict of interest.

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